

Immunogenicity of a killed *Leishmania* vaccine with saponin adjuvant in dogs

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Abstract

Cellular and humoral immune responses of dogs to a candidate vaccine, composed of *Leishmania braziliensis* promastigote protein plus saponin as adjuvant, have been investigated as a pre-requisite to understanding the mechanisms of immunogenicity against canine visceral leishmaniasis (CVL). The candidate vaccine elicited strong antigenicity related to the increases of anti-*Leishmania* IgG isotypes, together with higher levels of lymphocytes, particularly of circulating CD8⁺ T-lymphocytes and *Leishmania chagasi* antigen-specific CD8⁺ T-lymphocytes. As indicated by the intense cell proliferation and increased nitric oxide production during *in vitro* stimulation by *L. chagasi* soluble antigens, the candidate vaccine elicited an immune activation status potentially compatible with effective control of the etiological agent of CVL. © 2007 Elsevier Ltd. All rights reserved.

Keywords: Canine visceral leishmaniasis; Saponin adjuvant; Cell and humoral immune response

1. Introduction

Canine visceral leishmaniasis (CVL) is caused by *Leishmania* (*L.*) *chagasi* (syn. *Leishmania* (*L.*) *infantum*) and represents a major veterinary and public health problem in various regions of the New World and in countries around the Mediterranean basin [1]. In the endemic areas of Brazil, the

prevalence of CVL reportedly ranges from 5 to 35% [2]. The dog is the most important domestic reservoir of the etiological agent of the human visceral leishmaniasis, and for this reason the current strategy for managing the disease in man includes the detection and elimination of seropositive dogs alongside vector control and therapy for individual cases [3]. Thus, in the past 5 years the Brazilian Ministry of Health has directed the screening of around two million dogs and the elimination of more than 160,000 seropositive animals, but the incidence of human VL has not been reduced to an acceptable level [4]. These approaches have not been entirely effective, however, partly because of the resistance by dog owners to acquiesce in the euthanasia of their infected pets [5,6].

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In this context, a vaccine against VL would be an important tool in the control of CVL, and would also dramatically decrease the infection pressure of *L. chagasi* for humans [7]. Although an effective vaccine against human VL is not yet available, much effort has been expended in this area in recent years and several candidate vaccine antigens have been studied extensively in mice [8–14]. However, results obtained with a vaccine against VL that has been designed and tested using a mouse model cannot necessarily be extrapolated to other species [15]. This situation is well-illustrated by reference to the vaccine developed by Dunan et al. [16], which was effective in murine models but offered no protection against CVL [17]. Ideally, a vaccine designed to protect dogs should be developed using a canine model.

A recent strategy for the development of a vaccine against leishmaniasis has been based on the use of purified fractions from parasite extracts (FLM antigen) or from parasite cultures (excreted/secreted antigens), and some encouraging results have been reported [18–21]. However, vaccines prepared from whole parasites antigenic extracts still remain a reliable perspective considering their broad spectrum of antigenicity, cost and safety, and a number of such vaccines have been tested [22,23]. In phase I and II clinical trials, Mayrink et al. [24] demonstrated enhanced lymphocyte proliferation and significant protection against infection by *Leishmania* in Brazilian dogs that had received merthiolated, ultrasound-disrupted promastigotes of *L. braziliensis* together with Bacillus Calmete-Guérin (BCG). Strong cellular proliferation to soluble *Leishmania* antigens (SLA) has also been reported in dogs vaccinated with autoclaved *L. major* promastigotes (ALM) plus BCG [25]. Additionally, a vaccine composed of *L. infantum* promastigotes that had been freeze/thawed and then emulsified with Freund's complete adjuvant, induced high parasitocidal activity and increased the formation of nitric oxide (NO) in the macrophages of treated dogs [26]. Moreover, a single dose of a vaccine composed of aluminium hydroxide (alum)-precipitated *L. major* (alum-ALM) plus BCG has been shown to be safe and to decrease the incidence of CVL from 12 to 3.7%, which is equivalent to a 69.3% efficacy rate [27].

Considering the promising results obtained using *Leishmania* crude antigen vaccines [26], together with the somewhat simpler facilities required in their manufacture and the lower production costs involved, a killed *Leishmania* crude antigen vaccine could be useful in the control of CVL in endemic areas of developing countries. However, in most of the studies published, the detailed immune status of the dogs following vaccination was not evaluated, probably owing to the lack of specific reagents and standardised methods by which to investigate canine cell biology. In the present paper, we present a detailed analysis of the immunogenicity/antigenicity of a CVL vaccine composed of *L. braziliensis* antigens plus saponin as adjuvant.

2. Material and methods

Details of the study were presented to and approved by the Ethical Committee for the Use of Experimental Animals of the Universidade Federal de Minas Gerais, Belo Horizonte-MG, Brazil.

2.1. Design of vaccine

Promastigotes of *L. braziliensis* (MHOM/BR/75/M2903) were maintained in *in vitro* culture in NNN/LIT media as described previously [24]. Parasites were harvested by centrifugation ($2000 \times g$, 20 min, 4 °C) from 10-day-old cultures, washed three times in saline buffer, fully disrupted by ultrasound treatment (40 W, 1 min, 0 °C), separated into aliquots and stored at -80 °C until required for use. Protein concentration was determined according to the method of Lowry et al. [28]. The vaccine described is registered at the Instituto Nacional da Propriedade Industrial (Brazil) under patent number PI 0601225-6 (17 February 2006).

2.2. Study animals and vaccination

Twenty-five male and female mongrel dogs that had been born and reared in the kennels of the Instituto de Ciências Exatas e Biológicas, Universidade Federal de Ouro Preto, Ouro Preto, Minas Gerais, Brazil, were treated at 7 months with an anthelmintic and vaccinated against rabies (Tecpar, Curitiba-PR, Brazil), canine distemper, type 2 adenovirus, coronavirus, parainfluenza, parvovirus and leptospira (Vanguard® HTLP 5/CV-L; Pfizer Animal Health, New York, NY, USA). The absence of specific anti-*Leishmania* antibodies was confirmed by indirect fluorescence immunoassay.

Experimental dogs were treated within four experimental groups as follows: (i) control group C ($n = 10$) received 1 ml of sterile 0.9% saline; (ii) LB group ($n = 5$) received 600 µg of *L. braziliensis* promastigote protein in 1 ml sterile 0.9% saline; (iii) Sap group ($n = 5$) received 1 mg of saponin (Sigma Chemical Co., St. Louis, MO, USA) in 1 ml sterile 0.9% saline; and (iv) LBSap group ($n = 5$) received 600 µg of *L. braziliensis* promastigote protein and 1 mg of saponin in 1 ml sterile 0.9% saline. In each case animals received three subcutaneous injections in the right flank at intervals of 4 weeks.

2.3. Local and/or general reactions upon immunisation

Dogs were monitored closely for 2 weeks after each injection. General tolerance to vaccination was ascertained from an overall evaluation, including rectal temperature measurements, of the health of the animal. Local tolerance was determined by direct visual examination and any lesions observed were measured at 24 h intervals over a period of 72 h after each injection. All animals were followed up during the complete course of the study by a veterinarian who provided full medical support as required.

2.4. Blood sample collection

Peripheral blood (5 ml) was collected from the jugular vein of each dog and transferred to tubes containing sufficient EDTA to produce a final concentration of 1 mg/ml. The absolute count of lymphocytes in each sample was obtained using a Coulter (Miami, FL, USA) model MD18 instrument. Blood samples were stored at room temperature for up to 12 h prior to processing.

2.5. Humoral immune response

Immunogenicity was evaluated by the determination of antibodies against a soluble lysate of *L. chagasi* antigen (MHOM/BR/1972/BH46) (SLcA) according to the conventional enzyme-linked immunosorbent assay (ELISA) described by Reis et al. [29,30]. SLcA was coated onto 96-well microplates (MaxiSorp™, Nalge Nunc Intl., Rochester, NY, USA) at a concentration of 10 µg/well, the serum samples were added at 1:80 dilution, the wells were washed and peroxidase-conjugated goat anti-dog IgG1 or sheep anti-dog IgG and IgG2 (Bethyl Laboratories Inc., Montgomery, TX, USA) added at dilutions of 1:1000, 1:8000 and 1:16,000, respectively. The wells were then washed, substrate and chromogen (*o*-phenylenediamine; Sigma–Aldrich Co., St. Louis, MO, USA) added, and the absorbance read at 492 nm on a Multiskan® MCC 340 (Labsystems, Helsinki, Finland) automatic microplate reader.

2.6. Immunophenotyping

Unlabelled canine monoclonal antibodies (mAbs) anti-CD5 (rat-IgG2a: clone YKIX322.3), anti-CD4 (rat-IgG2a: clone YKIX302.9), anti-CD8 (rat-IgG1: clone YCATE55.9) were used in an indirect immunofluorescence procedure in which pooled normal rat serum (diluted 1:6000) was employed as isotypic control and fluorescein isothiocyanate (FITC)-labelled IgG sheep anti-rat polyclonal antibody was used as the secondary antibody. Non-specific binding of the second-step reagent was blocked with pooled normal sheep serum in phosphate-buffered saline (PBS) containing 10% of foetal bovine serum (Gibco, Grand Island, NY, USA).

FITC-labelled mouse anti-human-CD21 (mouse-IgG1: clone IOB1a), phycoerythrin (PE)-Cy5-conjugated mouse anti-human-CD14 (mouse-IgG2a: clone TÜK4), and RPE-conjugated mouse anti-mouse MHC-I (mouse-IgG2b: clone 2G5) mAbs were used in a direct immunofluorescence procedure. In an attempt to identify optimal dilutions for each assay, mAbs were previously titred in a solution of PBS containing 1% bovine serum albumin and 0.1% sodium azide. Unlabelled mAbs, anti-CD14 and anti-MHC-I mAbs were purchased from Serotec (Oxford, UK) and anti-CD21 was from Immunotech Co. (Marseille, France).

Microplate assays for immunophenotyping canine whole blood leukocytes (WBL) in both fresh blood samples and peripheral blood mononuclear cells (PBMC) obtained after

in vitro stimulation were carried out as described by Reis et al. [31].

2.7. Flow cytometry

Flow-cytometric measurements were performed on a FACScan instrument (Becton Dickinson, Mountain View, CA, USA) interfaced to an Apple G3 workstation. Cell-Quest software (Becton Dickinson) was used in both data acquisition and analysis. A total of 15,000 events were acquired for each preparation. Canine WBL were selected on the basis of their characteristic forward (FSC) and side (SSC) light-scatter distributions. Following FSC and SSC gain adjustments, the lymphocytes were selected by gating on the FSC versus SSC graph. Fluorescence was evaluated from FITC and PE-Cy5 spectra on FL1 or FL3 single-histogram representations. Monocytes were analysed by fluorescence intensity detection on single histograms obtained directly from ungated leukocytes. For data analysis, a marker was set as an internal control for non-specific binding in order to encompass >98% of unlabelled cells: this marker was then used in all data analysis for a given animal. The results were expressed as the percentage of positive cells within the selected gate for cell surface markers presenting bimodal distribution (CD5, CD4, CD8 and CD21). Semi-quantitative analyses were carried out for the cell surface marker (MHC-I) that exhibited a unimodal distribution in order to evaluate differential expression, and the results were expressed as mean fluorescence channel (MFC) on a log scale. Data were also expressed as absolute counts in order to allow the normalization of values obtained from groups whose overall leukocyte counts were different. The absolute counts for lymphocytes and monocytes were calculated as: (global leukocyte counts × percentage of lymphocytes or monocytes in hematology)/100. The absolute counts for lymphocyte subsets and monocytes were further calculated as: (absolute lymphocyte counts × percentage of fluorescent positive cells within lymphogate)/100 and (global leukocyte counts × percentage of fluorescent positive cells within ungated monocytes)/100.

2.8. *In vitro* assays

PBMC were isolated from 20 ml samples of heparinised blood that had been layered onto 10 ml of Ficoll–Hypaque density gradient (Histopaque® 1.077; Sigma Chemical Co.) and centrifuged at 450 × *g* for 40 min at room temperature. The separated PBMC were resuspended in Gibco RPMI 1640 medium, homogenised, washed twice with RPMI 1640, centrifuged at 450 × *g* for 10 min at room temperature, homogenised and finally resuspended in RPMI 1640 at 10⁷ cells/ml.

For in the *in vitro* assays, the cell culture medium comprised RPMI 1640 supplemented with streptomycin (100 mg/ml), penicillin (100 U/ml), L-glutamine (2 mM), β-mercaptoethanol (5 × 10⁻⁵ M) and 10% heat-inactivated foetal calf serum. The lymphoproliferation assays were

performed in 96-well flat-bottomed tissue culture plates (Corning, New York, NY, USA), each well containing 150 μ l of supplemented RPMI medium. Aliquots (25 μ l) of PBMC (2.5×10^5 cells/well) were added to triplicate wells together with 25 μ l of vaccine soluble antigen (VSA; *L. braziliensis*, 25 μ g/ml) or 25 μ l of SLcA (25 μ g/ml), obtained according to Reis et al. [29,30], for the antigenic stimulus assays. For the mitogenic stimulus assays, 25 μ l aliquots of PBMC (2.5×10^5 cells/well) were added to triplicate wells together with 25 μ l of phytohaemagglutinin (PHA; 2.5 μ g/ml; Sigma–Aldrich Chemie GmbH, Taufkirchen, Germany). Incubations were carried out in a humidified 5% CO₂ atmosphere at 37 °C for 3 days (mitogenic-stimulated cultures) or 5 days (antigenic-stimulated cultures). Six hours prior to the termination of the culture, 1 μ Ci of ³H-thymidine (Sigma Chemical Co.) was added to each well and the cells were subsequently harvested onto glass fibre filters. The incorporation of radioactivity was determined by liquid scintillation counting. Control assays were prepared exactly as above, employing 25 μ l aliquots of PBMC (2.5×10^5 cells/well) but with 25 μ l of RPMI 1640 medium replacing the stimulant, and were incubated for the appropriate time. Proliferation responses were expressed in terms of mean counts per minute in triplicate wells, whilst the stimulation index was calculated as: (mean proliferation response of cultures stimulated by VSA or SLcA/mean proliferation response of unstimulated cultures).

In order to investigate the immunophenotypic features, PBMC were cultured in 48-well flat-bottomed tissue culture plates (Costar, Cambridge, MA, USA), each well containing 650 μ l of supplemented RPMI medium. Aliquots (50 μ l) of PBMC (5.0×10^5 cells/well) were added to triplicate wells together with 100 μ l of VSA (25 μ g/ml) or 100 μ l of SLcA (25 μ g/ml). Control assays were prepared as above but employing 50 μ l aliquots of PBMC (5.0×10^5 cells/well) and 100 μ l of RPMI 1640 medium replacing the stimulant. Incubation was carried out in a humidified 5% CO₂ atmosphere at 37 °C for 5 days, after which the PBMC were removed for immunophenotyping and the supernatants were collected for further assay as described below.

2.9. NO levels

As an indirect measurement of NO production *in vitro*, nitrite levels were determined in the supernatants of PBMC cultures using the Griess reaction [32,33]. Briefly, a 100 μ l aliquot of Griess reagent (0.1% naphthylethylenediamine dihydrochloride, 1% sulphanylamide and 5% phosphoric acid; Sigma Chemical Co.) was added to the culture supernatant and, following 10 min incubation in the dark at room temperature, the absorbance was measured at 540 nm in an automatic microplate reader. Each sample was assayed in duplicate and the concentration of nitrite was determined by interpolation from a standard curve constructed using sodium nitrite solutions of known concentration in the range 0–100 μ mol/l. Data were expressed as means of the NO pro-

duction index (antigen-stimulated culture/control culture) in order to compare values in culture supernatants prepared at T0 (immediately prior to the application of the first dose of vaccine) and T3 (15 days after the application of the third dose of vaccine).

2.10. Statistical analyses

Statistical analyses were performed using Prism 4.0 software package (Prism Software, Irvine, CA, USA). Normality of the data was demonstrated using a Kolmogorov–Smirnov test. One-way analysis of variance (ANOVA) and Tukey post-tests were used for determining the differences between groups in terms of humoral immune responses and immunophenotypic profiles. Student's *t*-tests were used to evaluate differences in mean values determined in *in vitro* assays of stimulated cultures and control cultures prepared at T0 and T3. Pearson's rank correlation was employed to investigate associations between phenotypic features in circulating leukocytes or between phenotypic features and cell proliferation. In all cases, differences were considered significant when *P* values were <0.05.

3. Results

3.1. Local induration in dogs that had received saponin as adjuvant represented the major adverse reaction observed

Vaccination was not associated with hyperthermia, pain, fever, lymphadenopathy or any other general adverse reactions. Moreover, no local adverse reactions were observed in vaccinated dogs, with the exception of mild local induration reactions in some dogs vaccinated with preparations containing saponins (Table 1). Such nodules were most commonly observed after the second injection of vaccine, but did not result in the formation of ulcerated lesions.

Table 1
Local alterations in the inoculum region measured 72 h after saponin or *L. braziliensis* vaccine + saponin inoculation

Group	Animal code	Nodule size (cm)		
		T1 ^a	T2 ^b	T3 ^c
Sap (n = 5)	C03	–	–	–
	C05	2.5 × 1.5	4.0 × 4.5	–
	C14	–	–	–
	C20	–	2.0 × 3.0	2.0 × 2.0
	C27	–	–	–
LBSap (n = 5)	C04	–	2.0 × 2.0	–
	C30	–	–	–
	C36	–	–	–
	C12	–	–	5.0 × 4.0
	C19	–	–	–

^a T1: 72 h after the first dose.

^b T2: 72 h after the second dose.

^c T3: 72 h after the third dose.

3.2. LBSap elicited an intense immunogenic reaction that was characterised by elevated levels of IgG1 and IgG2 antibodies to *Leishmania*, the association of which signified a mixed Th1/Th2 immune response

Significant ($P < 0.05$) increases in the serum levels of anti-*Leishmania* total IgG at T2 (15 days after the application of the second dose of vaccine) and T3 were observed in dogs of the LBSap group compared with those of the Sap, LB and control (C) groups (Fig. 1, upper left panel). Further analysis demonstrated that the levels of both anti-*Leishmania* IgG1 (Fig. 1, upper right panel) and IgG2 (Fig. 1, lower left panel) were enhanced ($P < 0.05$) in the LBSap group at both T2 and T3 compared with the other groups. Moreover, there was a positive association between IgG1 and IgG2 levels (Fig. 1, lower right panel).

3.3. LBSap elicited an increase in the numbers of circulating CD5⁺ T-lymphocytes, mainly as CD4⁺ and CD8⁺ T-cell subsets, and CD21⁺ B-lymphocytes

A preliminary comparative analysis of the cell profiles of the treatment groups showed that at T1 (15 days after the application of the first dose of vaccine) there was a sig-

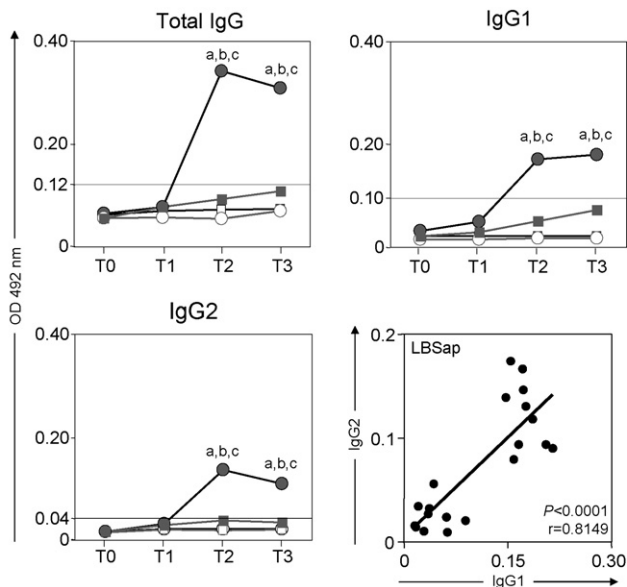


Fig. 1. Comparative immunogenicity in the different treatment groups: C (control; □); Sap (saponin; ○); LB (killed *L. braziliensis* vaccine; ■); LBSap (killed *L. braziliensis* vaccine plus saponin; ●). Upper left and right panels and lower left panel show anti-*L. chagasi* total IgG, anti-*L. chagasi* IgG1, and anti-*L. chagasi* IgG2, respectively: the x-axis displays the times at which the assays were conducted (T0: prior to the first dose; T1: 15 days after the first dose; T2: 15 days after the second dose; and T3: 15 days after the third dose) and the y-axis represents the mean ELISA absorbance values determined at 492 nm in serum samples diluted 1:80. Significant differences ($P < 0.05$) between the LBSap group and the control C, Sap, and LB groups are indicated, respectively, by the letters a, b, and c. The lower right panel shows the correlation between IgG1 and IgG2 in the LBSap group and the Pearson's correlation indexes (r) at $P < 0.05$ are shown in figure.

nificant ($P < 0.05$) reduction in the numbers of circulating lymphocytes in the Sap and LB groups compared with the control group C (Fig. 2, upper panel). Detailed investigation of the immunophenotypic features of the lymphocytes (Fig. 2, middle panels) revealed that at T1 there was an increase ($P < 0.05$) in the number of circulating CD5⁺ T-lymphocytes in the LBSap group compared with the Sap group, and significantly ($P < 0.05$) higher counts of both CD4⁺ and CD8⁺ T-cell subsets and CD21⁺ B-cells in the LBSap group compared with the Sap and control C groups. Furthermore, from the absolute counts obtained at T1, T2 and T3, it was possible to demonstrate positive correlations within the LBSap group with respect to CD5⁺ T-cells versus CD21⁺ B-cells ($P = 0.0024$; $r = 0.6395$), and CD8⁺ versus CD4⁺ T-cells ($P < 0.0001$; $r = 0.8533$) (Fig. 2, middle panels), and with respect to CD4⁺ ($P = 0.0002$; $r = 0.7422$) and CD8⁺ ($P < 0.0001$; $r = 0.8420$) versus CD5⁺ T-cells (Fig. 2, lower panel).

3.4. In vitro cell proliferation in the presence of antigenic stimuli was increased intensely following vaccination with LBSap but reduced significantly after treatment with LB

In vitro cell reactivities were determined in the presence of the VSA in order to evaluate memory lymphoproliferative immune response and in the presence of SLcA in order to investigate possible lymphoproliferative homology with the complete antigenic repertoire of the etiological agent of VL (Fig. 3, upper panel). The lymphoproliferative activity at T3 compared with that at T0 in the LB group was reduced ($P < 0.05$) in the presence of either stimulus. In contrast, the LBSap group displayed significant ($P < 0.05$) increases in stimulation index at T3 compared with T0 in the presence of both stimuli. Additionally, at T3 the values recorded in the presence of VSA was significantly ($P < 0.05$) higher than those observed in the LB, Sap and control C groups. Analysis of lymphoproliferative immune response and in the presence of SLcA also demonstrated at T3 a significant increase ($P < 0.05$) in comparison to that observed in the LB group.

The non-specific lymphoproliferative response was evaluated as stimulation index (SI) of PHA mitogen-induced cell proliferation. Our data confirmed the high degree of cell viability as demonstrated by outstanding SI values observed in the mitogenic-stimulated cultures that ranged from mean values of 8 to 15 (data not shown).

3.5. Vaccination with LBSap increased the frequencies of CD21⁺ B-lymphocytes and CD8⁺ T-lymphocytes and decreased the levels of CD4⁺ T-cells in antigen-stimulated in vitro cell proliferation cultures

In vitro immunophenotypic profiles at T3 were evaluated in the presence and absence of VSA or SLcA. In the presence of VSA (Fig. 3, left middle and lower panels), sig-

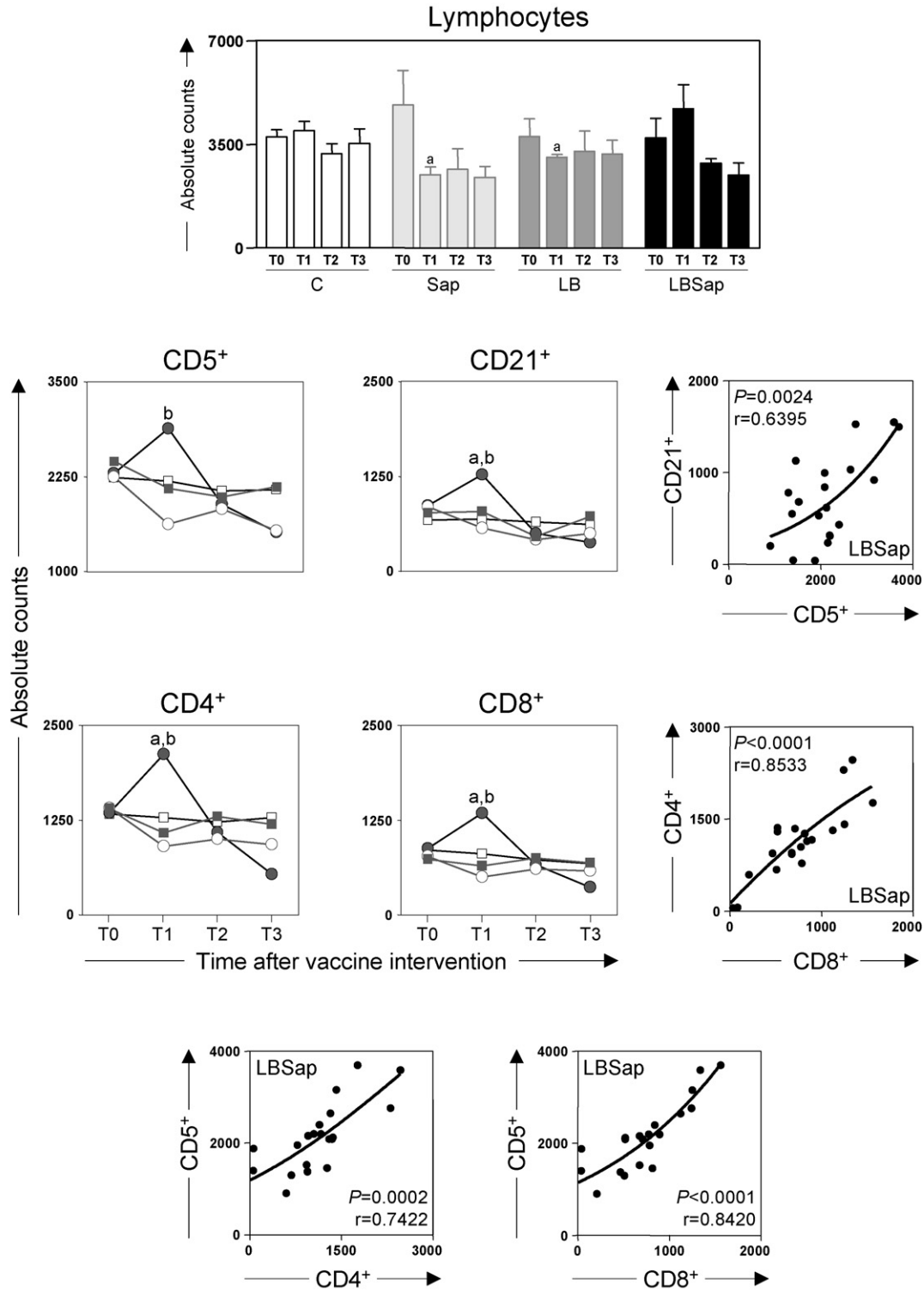


Fig. 2. The cell profile of peripheral blood leucocytes in different treatment groups. Upper panel [C (control; □); Sap (saponin; □); LB (killed *L. braziliensis* vaccine; ■), LBSap (killed *L. braziliensis* vaccine plus saponin; ■)] and middle left panels [C (control; □); Sap (saponin; ○); LB (killed *L. braziliensis* vaccine; ■); LBSap (killed *L. braziliensis* vaccine plus saponin; ●)]: the x-axis displays the times at which the assays were conducted (T0: prior to the first dose; T1: 15 days after the first dose; T2: 15 days after the second dose; and T3: 15 days after the third dose) and the y-axis represents the mean values (with standard deviations in the upper panel) of the absolute counts of circulating lymphocytes (upper panel), and of CD5⁺, CD21⁺, CD4⁺ and CD8⁺ cells (middle left panels). Significant differences ($P < 0.05$) between the LBSap group and the control C and Sap groups are indicated, respectively, by the letters a, b. The middle right panels and the lower panels show the correlations between circulating lymphocytes in the LBSap group and the Pearson's correlation indexes (r) at $P < 0.05$ are shown in figure.

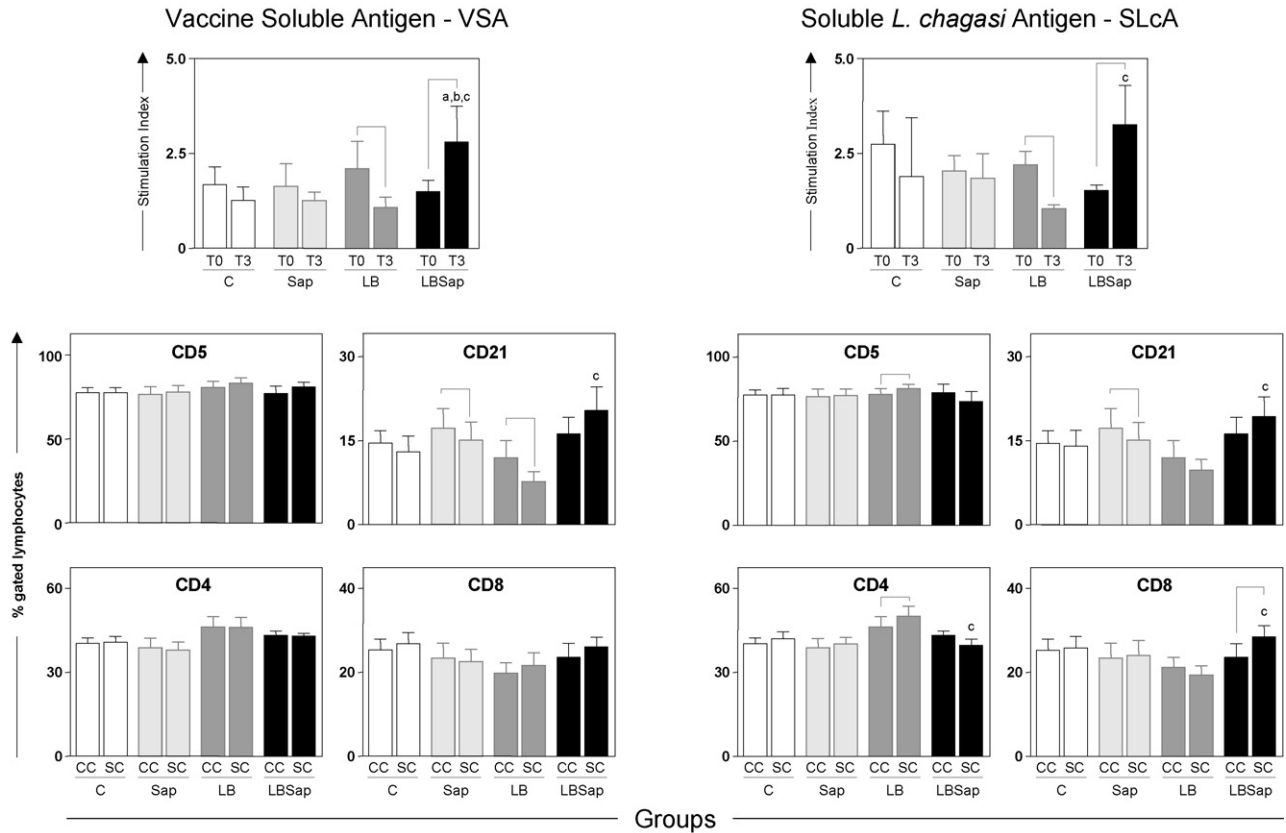


Fig. 3. Cell proliferation response of peripheral blood mononuclear cells after stimulation with vaccine soluble antigen (VSA) (upper left panel) and soluble *L. chagasi* antigen (SLcA) (upper right panel). The middle and lower panels show the immunophenotypic profile of *in vitro* peripheral blood mononuclear cells following stimulation with vaccine soluble antigen (VSA) (left panels) and soluble *L. chagasi* antigen stimulation (right panel) determined at T3 for treatment groups: C (control; □); Sap (saponin; ▤); LB (killed *L. braziliensis* vaccine; ▨); LBSap (killed *L. braziliensis* vaccine plus saponin; ■). The results are expressed as the mean frequencies of CD5⁺, CD21⁺, CD4⁺ and CD8⁺ cells in the non-stimulated cultures (CC; controls) and in the stimulated cultures (SC). Significant differences ($P < 0.05$) between values measured at T0 (before the first dose) and T3 (15 days after the third dose) are indicated by connecting lines, and between the LBSap and the control C, Sap, and LB groups at T3 are represented by the letters a, b, and c, respectively.

nificantly ($P < 0.05$) reduced frequencies of CD21⁺ B-cells were observed in the Sap and LB groups compared with those in the non-stimulated control cultures. Moreover, following stimulation with VSA, the LBSap group exhibited a higher ($P < 0.05$) frequency of CD21⁺ B-lymphocytes compared with the LB group. Similar results were obtained when *in vitro* cultures were stimulated with SLcA (Fig. 3, right middle and lower panels), indicating that the frequency of CD21⁺ B-cells was decreased ($P < 0.05$) in the Sap group compared with the control cultures, but increased ($P < 0.05$) in the LBSap group compared with the LB group. Moreover, although no differences had been observed in the frequencies of T-cells in assays involving VSA stimulation, when cultures were stimulated with SLcA, the LB group exhibited a higher frequency of CD5⁺ T-cells compared with the control C group, and this was mainly due to increased ($P < 0.05$) levels of CD4⁺ T-cells. Additionally, compared with the LB group, the LBSap group presented lower ($P < 0.05$) levels of CD4⁺ T-cells and an increased ($P < 0.05$) frequency of CD8⁺ T-cells.

3.6. LBSap vaccination gave rise to an increase in the levels of circulating CD14⁺ monocytes and an associated up-regulation of MHC-I expression by lymphocytes

In the search for potential antigen-presenting cells (APC) in animals that had been vaccinated with LBSap, it was observed that the numbers of circulating CD14⁺ monocytes were higher ($P < 0.05$) in this group of dogs than in the Sap group at T2, and in the LB and Sap groups at T3 (Fig. 4, upper left panel). Analysis of the data relating to the status of lymphocyte activation demonstrated a differential expression of MHC-I in the LBSap group, leading to a significantly ($P < 0.05$) increased mean fluorescence channel (MFC) in gated lymphocytes, compared with the LB, Sap and control C groups at T2, and the LB group at T3 (Fig. 4, upper middle panel). Further investigation revealed a positive association between lymphocyte activation status (MHC-I) and the level of circulating CD14⁺ monocytes ($P = 0.0212$; $r = 0.6075$), highlighting the connectivity of events during vaccine immunisations (Fig. 4, upper right panel).

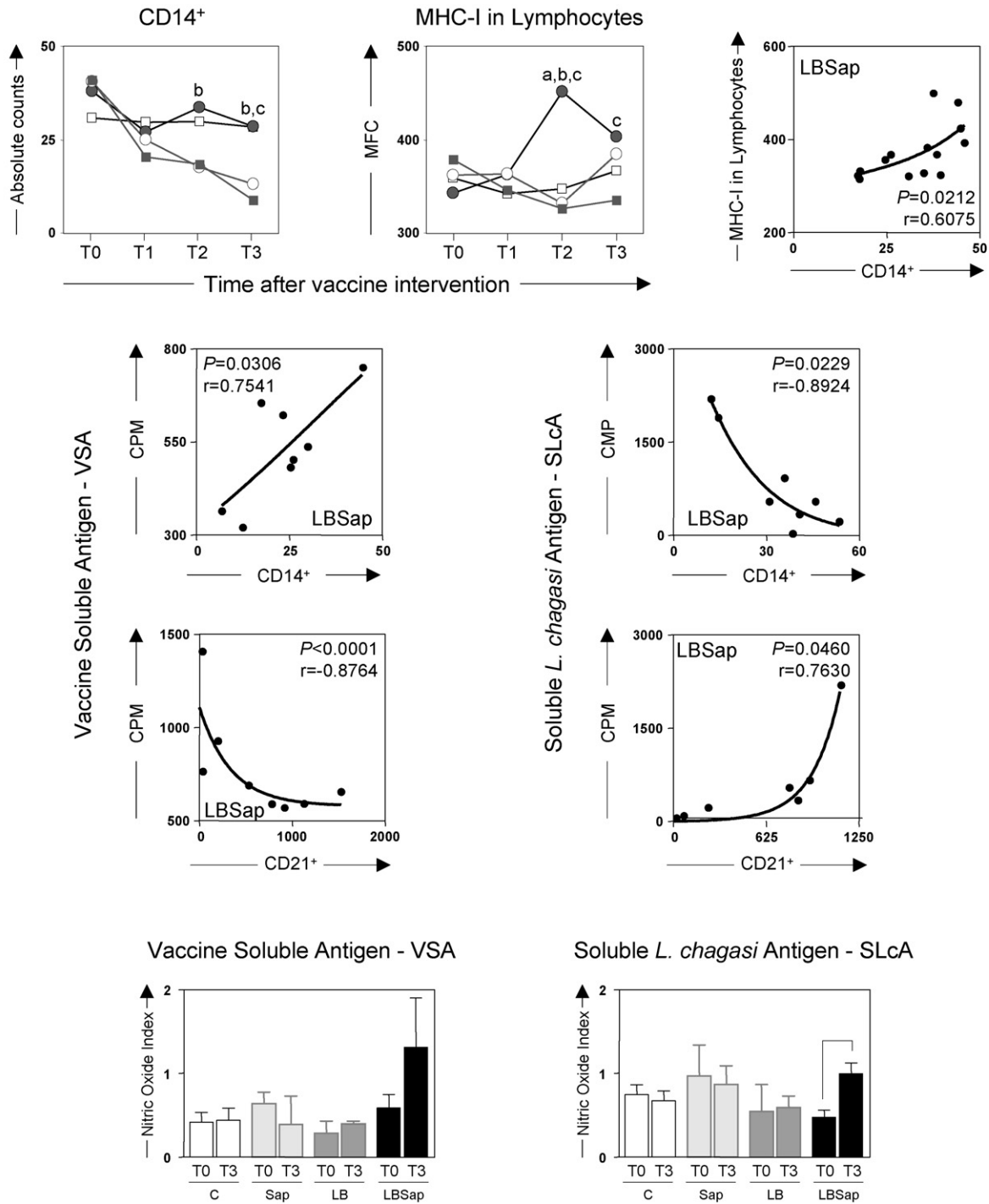


Fig. 4. Antigen-presenting cells (APC) and the lymphocyte activation status in different vaccine groups: C (control; □); Sap (saponin; ○); LB (killed *L. braziliensis* vaccine; ■); LBSap (killed *L. braziliensis* vaccine plus saponin; ●). In the upper left panels, significant differences ($P < 0.05$) between the LBSap group and the control C, Sap, and LB groups with respect to the absolute counts of CD14⁺ monocytes and MHC-I expression in lymphocytes (reported as MFC values) are indicated, respectively, by the letters a, b, and c. The correlation between CD14⁺ cell counts and MHC-I in lymphocytes in the LBSap group at T0, T1, T2 and T3 is shown in the upper right panel and the Pearson's correlation indexes (r) at $P < 0.05$ are shown in figure. The middle panels show the correlations between CD14⁺ and CD21⁺ absolute cell counts and *in vitro* cell proliferation (counts per minutes – CPM) following stimulation by vaccine soluble antigen (VSA) or soluble *L. chagasi* antigen (SLcA) and the Pearson's correlation indexes (r) at $P < 0.05$ are shown in figure. The lower panels display NO production indices (antigen-stimulated culture/control culture) determined at T0 and T3 in culture supernatants from the different treatment groups: C (control; □); Sap (saponin; ○); LB (killed *L. braziliensis* vaccine; ■); LBSap (killed *L. braziliensis* vaccine plus saponin; ●). Significant differences ($P < 0.05$) between at T0 plus T3 are indicated by connecting lines.

3.7. The correlations observed between cell proliferation in antigen-stimulated *in vitro* cultures and the levels of circulating CD14⁺ monocytes and CD21⁺ B-lymphocytes indicate the possibility of distinct APC profiles during LBSap vaccination

In order to identify which APC imparts the major contribution during specific *in vitro* antigenic stimulation, correlation analyses were performed using data derived from the LBSap group (Fig. 4, middle panel). The results demonstrate a positive association between CD14⁺ monocytes and *in vitro* cell proliferation in cultures stimulated with VSA ($P=0.0306$; $r=0.7541$), and a negative correlation in cultures stimulated with SLcA ($P=0.0229$; $r=-0.8924$). In contrast, CD21⁺ B-cell counts exhibited a negative association with cell proliferation in VSA-stimulated cultures ($P<0.0001$; $r=-0.8764$), and a positive association in cultures stimulated with SLcA ($P=0.0460$; $r=0.7630$). These data provide evidence for a distinct APC according to antigen stimulus, and suggest that CD14⁺ monocytes might act as APC in vaccine antigenic stimulation whilst CD21⁺ B-cell could fulfil this role in SLcA stimulation.

3.8. LBSap enhanced NO production in SLcA-stimulated *in vitro* cell proliferation cultures

Since the production of NO is considered to be a key element in killing mechanisms that mediate the elimination of intracellular pathogens, the levels of anti-microbial oxidant produced by *in vitro* antigenic-stimulated PBMC derived from dogs vaccinated with LBSap were determined. Interestingly, higher ($P<0.05$) levels of the reactive NO radical (nitrite) were recorded in the supernatant of SLcA-stimulated cultures at T3 compared with T0 (Fig. 4, lower panel), suggesting an outcome related to the Th1 immune response.

3.9. Positive correlations were observed between cell proliferation in antigen-stimulated *in vitro* PBMC cultures and the levels of CD4⁺ and CD8⁺ T-lymphocytes only in the LBSap group

In an attempt to determine whether the immunophenotypic features of *in vitro* PBMC cultures subjected to antigen stimulation were associated with a specific cell profile, we analysed the levels of association between cell type and proliferation within all four groups (Fig. 5). Data analysis demonstrated positive associations only in the LBSap group, and specifically between CD4⁺ and CD8⁺ T-cells and cell reactivity in both VSA- and SLcA-stimulated cultures.

4. Discussion

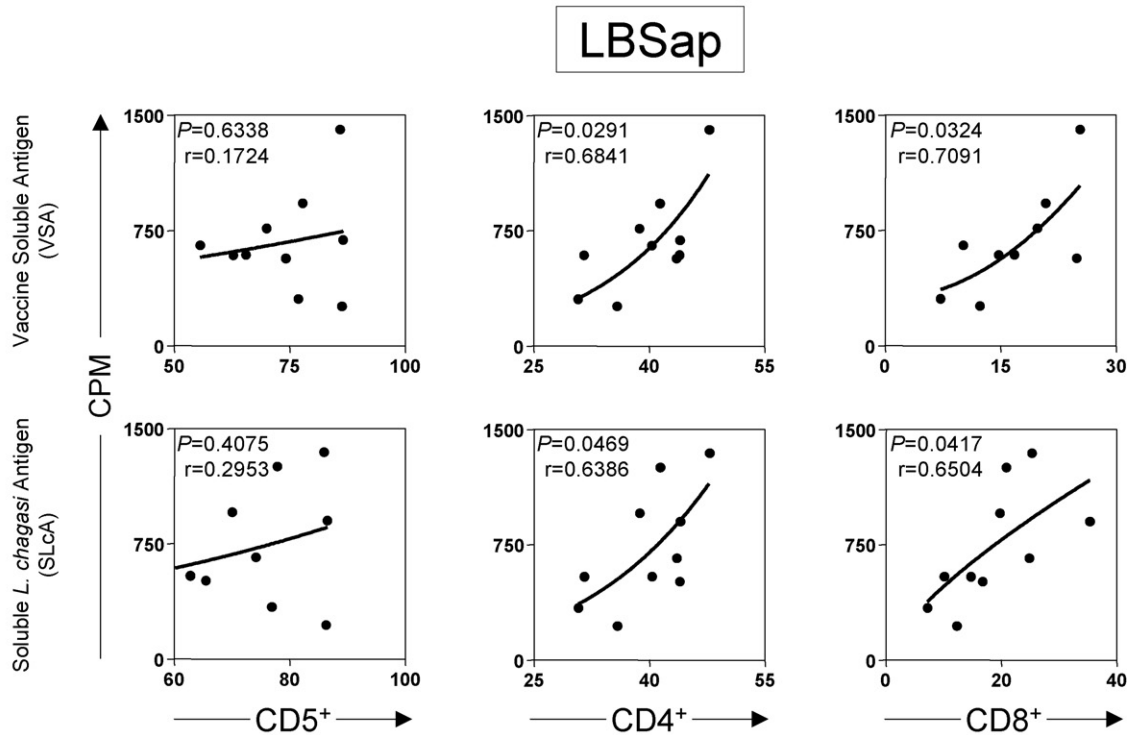
Canine visceral leishmaniasis, which resembles human visceral leishmaniasis with respect to many of its symptoms, is a severe chronic disease that is often fatal [29,34]. The

natural history of CVL has been well described, particularly in regard to the parasite load in different tissues and the immunopathological changes according to progression of clinical forms [29,30,35–37]. These data provide support for the improvement of the tools employed in the evaluation of both chemotherapies and vaccines that have been developed for CVL. Unfortunately, different treatment strategies have failed to achieve a consistent parasitological cure for CVL owing to the presence of latently infected cells [38,39]. In this context, a dog vaccine may be the most practical and effective method by which to reduce the incidence of human VL, and it might also permit a similar vaccine to be developed for humans [12,14,15,40].

All of these features point to immunoprophylaxis as a promising alternative for prevention of CVL. For this reason a considerable effort has been dedicated to studies on immune responses in CVL, and several *Leishmania* antigens implicated in these responses have been reported [14,15,41]. There is a major consensus that *L. chagasi/infantum* antigens display a potent immunosuppressive potential that would be deleterious for the immunoprotection against CVL. Several studies have reported the potential of *L. chagasi* antigens to trigger immunosuppression by blocking the *in vitro* lymphoproliferative response to *Leishmania* antigens as well as the synthesis of pro-inflammatory cytokines by antigen-presenting cells [42,43]. The use of purified *Leishmania donovani* and *L. infantum* antigens has been also proposed to overcome this immunosuppressive effect of *L. chagasi* antigens [18–20]. Most studies, including those clinical trials with vaccine candidates to CVL immunoprophylaxis have been conducted using either *L. amazonensis*, *L. braziliensis* or *L. major* antigens. Previous studies from our group have demonstrated that *L. braziliensis* antigen have a potent role in protecting *L. chagasi* infection in dogs (unpublished data). Therefore, a critical question for screening and development of anti-leishmanial vaccines in CVL is to define *Leishmania* antigens and adjuvant systems that elicit a favorable and sustained cytokine environment *in vivo*.

Considering the importance of immunoprophylaxis strategies for the control of leishmaniasis, and the lack of studies concerning the cellular and humoral events that occur during vaccination, we have attempted to evaluate the immune response of a promising new vaccine candidate against CVL composed of *L. braziliensis* antigens plus saponin as adjuvant. The assessment of such information is an essential pre-requisite to the understanding of mechanisms related to immunogenicity elicited by candidate vaccines.

The results obtained indicate that some dogs exhibit local swelling reactions, but no ulcerated lesions or other adverse reactions, after receiving saponin as an adjuvant. Although the overall tolerance of the candidate vaccine in dogs appeared to be adequate, it is important to mention that the frequency and/or size of local reactions cannot be itself sufficient as safety standards for veterinary vaccines. Therefore, further studies are still required to overcome this issue by searching for additional safety biomarkers for the use



Correlation indexes

Groups	CD5		CD4		CD8		
	VSA	SLcA	VSA	SLcA	VSA	SLcA	
C	r	0.1373	0.3469	-0.0675	-0.2823	-0.1089	-0.0048
	P	0.5752	0.1881	0.7771	0.2416	0.6476	0.9844
Sap	r	0.0841	0.4837	-0.3804	-0.3359	0.5179	0.4707
	P	0.8174	0.1566	0.2782	0.3426	0.1252	0.1697
LB	r	-0.4220	-0.2303	-0.2346	0.0967	0.0071	-0.3262
	P	0.2245	0.5221	0.5142	0.7904	0.9844	0.3916

Fig. 5. Correlations between cell proliferation (counts per minute) and frequencies of CD5⁺, CD4⁺ and CD8⁺ T-cells following *in vitro* peripheral blood mononuclear cell cultures derived from the LBSap group (upper panels), and C (control), Sap (saponin) and LB (killed *L. braziliensis* vaccine) groups (lower panel), stimulated by vaccine soluble antigen (VSA; upper panel) and soluble *L. chagasi* antigen (SLcA; middle panel) and determined at T0 (before the first dose) plus T3 (15 days after the third dose) and the Pearson's correlation indexes (*r*) at *P* < 0.05 are shown in figure.

of saponin as vaccine adjuvant. Some side effects that have been reported following the use of saponin adjuvants include some non-specific immune reactions, loss of hair at the site of injection, anorexia, apathy, vomiting and diarrhoea [44–46]. However, as saponin induces the development of strong CD8⁺ T-lymphocytes cytotoxicity [47], its use as adjuvant has been included in several veterinary vaccines [44].

In the present study, the evaluation of immunogenicity of LB and LBSap revealed that animals treated with LB together with saponin adjuvant presented higher (*P* < 0.05) amounts of anti-*Leishmania* total IgG that were associated

with increased (*P* < 0.05) levels of IgG1 and IgG2. Since IgG1 and IgG2 responses are strictly T-cell dependent, they have previously been employed as measures of the overall immunogenicity of recombinant antigens in dogs [48]. Furthermore, IgG1 and IgG2 subtypes have been traditionally used as surrogates of the Th2 and Th1 phenotypes of immune response in mice whereas IgG4 response is more generally related to a Th2 response in humans. However, the association between IgG subtypes and the immune response in terms of the Th1/Th2 phenotype is not so straightforward in dogs [30,49–52], the finding of both immunoglobulin isotypes sug-

gested that a mixed immune response is triggered by LBSap vaccination.

The intense humoral immune response demonstrated in the LBSap group was synchronous with increased ($P < 0.05$) counts of circulating CD21⁺ B-cells following the first immunisation, resulting in differentiation of plasmacytes and higher levels of immunoglobulin secretion. Additionally, the higher numbers ($P < 0.05$) of circulating CD5⁺ T-cells in the LBSap group were positively correlated with CD21⁺ B-cells suggesting a possible cooperation between T- and B-cells during the immunisation sequence.

Protection against infection by *Leishmania* relies on the cell-mediated immune response, which implies that a successful immunisation protocol should be able to activate cell-mediated immunity in the immunised animal [23,53,54]. In the present study, the increase in CD5⁺ T-cells in the LBSap group was associated with increased levels of CD4⁺ and CD8⁺ T-cells. Moreover, positive correlations between CD5⁺ T-cells and the CD4⁺ and CD8⁺ T-cell subset, and between CD4⁺ and CD8⁺ T-cells, further suggested significant cooperation at the cellular level as has been observed during early clonal expansion and the generation of primary CD8⁺ cytotoxic effectors [55]. Additionally, the augmentation of circulating CD8⁺ T-cells during on-going CVL has been previously described as the major phenotypic feature of the asymptomatic disease in dogs bearing low parasite loads [37]. Thus, our findings support the hypothesis that CD8⁺ T-lymphocytes play a role in protective immunity during *Leishmania* infection as has been suggested previously for CVL [37,56].

In order to evaluate the activation status of lymphocytes during the immunisation protocol, the expression of MHC-I was studied. The finding of a higher expression of MHC-I in lymphocytes in the LBSap group indicated an improvement in the activation status of lymphocytes during the immunisation protocol. It has been proposed that an increase in the expression of MHC-II may reflect an antigenic priming-related immunological event [37,57]. Consistent with this hypothesis, we have demonstrated that dogs with asymptomatic CVL displayed an enhanced activation status of circulating lymphocytes (as determined from MHC-II) [37], together with lower overall tissue parasitism [30,37]. However, data from the present study highlight the importance of MHC-I expression as an activation marker in lymphocytes. In fact, the up-regulation of MHC-I and -II are both related to the presence of IFN- γ [58,59]. Consistent with this hypothesis, the evaluation of APC revealed increased numbers of circulating CD14⁺ monocytes in the LBSap group that were associated with increased MHC-I expression in lymphocytes. On the basis of these results, it is possible to speculate that this association would represent the interactions between innate and adaptive immune responses, reflecting in improvement in activation status during LBSap immunisation.

Aiming to determine whether the candidate vaccine would activate PBMC under *in vitro* antigenic stimulation, we measured the stimulation index at T0 and T1 in cells derived from

LBSap and LB dogs. Higher cell reactivities following stimulation by either VSA or SLcA were recorded for the LBSap group after the third immunisation, whereas the stimulation index in the LB group was lower at T3 compared to T0. Thus, our findings support the hypothesis that PBMC proliferation in the LBSap group is associated with the recognition of *L. chagasi* antigen, and suggest that this vaccine may be of value against the etiological agent of VL.

Further analyses were carried out in order to investigate the profile of APC in *in vitro* cultures of PBMC from the LBSap group subjected to antigenic stimuli. Whilst cell proliferation exhibited a positive association with circulating CD14⁺ monocytes in the presence of VSA, a negative correlation was observed upon SLcA stimulation. In contrast, a negative association between CD21⁺ B-cells and cell proliferation was observed in the presence of VSA, and a positive association upon SLcA stimulation. These findings indicate a distinct profile of APC in the cultures and establish that CD14⁺ monocytes and CD21⁺ B-cells are the major APC, respectively, during *in vitro* stimulation with VSA and SLcA.

When *in vitro* cultures of PBMC derived from the LBSap group were stimulated with VSA or SLcA, increased lymphoproliferation activity was accompanied by a higher frequency of CD21⁺ B-cells demonstrating the potential immunoglobulin isotypes produced. In contrast, no differences were observed in the frequency of CD5⁺ T-cells following *in vitro* stimulation, whilst reductions in the frequency of CD4⁺ T-cells and increases in the level of CD8⁺ T-cells were associated with SLcA stimulus. These results support the hypothesis that CD8⁺ T-cells play a protective role in the mechanism of control of *Leishmania* parasitism. Additional correlation analysis revealed that both CD4⁺ and CD8⁺ T-cells exhibited positive associations with cell proliferation *in vitro* following stimulation with VSA or SLcA. These data suggest the possibility of stimulating antigen-specific T-cell subsets following LBSap immunisation that could contribute to improvements in the cellular immune response during *L. chagasi* infection.

The results obtained from the analysis of NO levels (determined as nitrite) in culture supernatants confirmed the hypothesis that LBSap immunisation induces a potential resistance profile against *Leishmania* infection despite the higher nitrite levels observed in *in vitro* T3 cells stimulated by SLcA.

In conclusion, the results presented in this study provide support for the continued development of vaccines based on the whole parasite approach. Such a strategy for vaccine design is attractive in terms of safety and stability of product compared with purified subunit preparations or DNA vaccines, both of which involve more sophisticated technology that may not be readily available in developing countries. Furthermore, in contrast to recombinant vaccines, killed vaccines require fewer tests to be carried out on the bulk intermediates and finished products, and this makes the cost of production lower than for recombinant proteins [60]. Additionally, killed vaccines present a great diversity

in antigenic repertoire, and this should potentially activate a stronger cellular response, mainly by T-lymphocytes, compared with that of purified subunit preparations or DNA vaccines.

The major findings in the present study point to a strong antigenicity of the candidate vaccine related to increased immunoglobulin isotypes, together with higher levels of lymphocytes, particularly in circulating CD8⁺ T-lymphocytes and SLcA-specific CD8⁺ T-lymphocytes. In addition, the intense cell proliferation and NO production during *in vitro* stimulation by SLcA shows that LBSap vaccine elicited a potential immune activation status potentially compatible with effective control of the etiological agent of CVL. Further investigations will focus on the efficacy of the LBSap vaccination in protection against experimental challenge with *L. chagasi*.

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